Association between PADI4 gene polymorphisms and anti-cyclic citrullinated peptide antibody positive rheumatoid arthritis in a large Chinese Han cohort

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Abstract

Objective

The present study was undertaken to investigate the association of peptidyl-arginine-deiminase type IV gene (PADI4) single nucleotide polymorphisms (SNPs) with rheumatoid arthritis (RA) susceptibility, and to determine whether there is any impact of PADI4 polymorphisms on RA subsets or phenotypes in a large Chinese Han cohort.

Methods

Two PADI4 SNPs (rs2240340 and rs1748033) were genotyped in 1216 Chinese Han RA patients and 1040 unaffected controls by TaqMan SNP Assays. Serum anti-CCP antibody and anti-PAD4 antibody levels were measured by ELISA. Bone destruction was scored by Sharp-van der Heijde scores (SHSs) of hands in 463 patients.

Results

The two SNPs rs2240340 and rs1748033 of PADI4 showed strong association with RA susceptibility (OR=1.23, 95% CI 1.09-1.38, $p=6.66\times10^{-4}$; and OR=1.24, 95% CI 1.10-1.41, $p=6.98\times10^{-4}$, respectively). RA risk genotypes of PADI4 were specifically associated with anti-CCP positive RA (rs2240340: $p=5.13\times10^{-6}$; rs1748033: $p=2.97\times10^{-3}$, respectively). Furthermore, there was a trend association between PADI4 rs2240340 and radiographic severity, though it did not reach the statistic significance (p=0.088).

Conclusion

Our data provide strong evidence that PADI4 polymorphisms are risk factors contributed to RA susceptibility, especially for anti-CCP positive RA, and may confer higher risk of RA radiographic severity in Chinese Han population.

Key words

rheumatoid arthritis, peptidyl-arginine-deiminase type IV, single nucleotide polymorphism, anti-cyclic citrullinated peptide antibody, bone destruction

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Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease with unknown etiology. It is characterised by consistent inflammation and joint destruction. Epidemiologic data have demonstrated that the genetic factors have strong influence on RA susceptibility (1, 2). The most putative RA genetic factors are the human leukocyte antigen (HLA) genes, which contributed about one third of the genetic component to RA susceptibility (3, 4). Besides, recent genetic studies have revealed multiple non-HLA susceptible genes for RA(5). Of which, the peptidylarginine deiminase 4 (*PADI4*) gene was reported to be RA risk factor based on the results of association studies from Japanese, Korean and other populations (6-11). However, the data from Chinese Han population were controversial, most likely due to the modest sample size in those studies (12, 13).

It has been accepted that anti-citrullinated protein antibodies (ACPA) are specific in RA and arise early in the disease course (14, 15). PAD4 belongs to PAD family which generates the citrullinated proteins recognised by ACPA via post-translational modification. Previous studies have shown that *PADI4* conferred greater risk for anti-cyclic citrullinated peptide antibodies (anti-CCP) -positive than for anti-CCP -negative RA (14, 16, 17). And *PADI4* polymorphisms were correlated with erosive disease in Japanese (the Sharp-van der Heijde scores at 5-year disease duration) and in Caucasians (Steinbrocker score >II) (18, 19). However, results were controversial in Korean populations, in which bone erosion was also assessed by Steinbrocker score (20). The aim of this work was to provide further evidence of *PADI4* polymorphisms as risk factor for RA susceptibility, to evaluate whether *PADI4* polymorphisms were specifically associated with any subsets of RA, based on RA serologic features, and to further investigate its influence on radiographic severity in RA patients in a large Chinese Han cohort.

Materials and methods

Selection of PADI4 SNPs

In present study, we proposed a candidate approach and 5 SNPs were selected. The 5 SNPs, flanking along exon 2, 3 and 4, have been extensively reported to be associated with RA both in Asians and in Caucasians (7, 21-23). Four of the 5 SNPs are coding SNPs and another is resided in intron 4-5 (rs2240340). We first preformed the association analysis between the 5 SNPs and RA in 220 cases and 224 healthy controls. As shown in Figure 1, the 5 SNPs were in strong linkage disequilibrium (LD) and constitute a single haplotype block (D'>0.95). Therefore, two SNPs rs2240340 (PADI4_94) and rs1748033 (PADI4_104) were further genotyped. The reason for further choosing the intronic SNP rs2240340 (PADI4_94) was that the SNP was the most extensively studied candidate in RA association previously (7, 18, 21-26).

Study subjects

There were 1216 RA patients (mean onset age 46.0±14.4 years; 81.4% females) and 1040 unrelated controls (mean age 40.8±16.3, years; 75.1% females) enrolled in our cohort. All patients met the 1987 American College of Rheumatology revised criteria of RA(27). In which, 82.5% (675/818) were anti-CCP positive RA. The control group comprised 1,040 unrelated healthy individuals (mean age 40.8±16.3 years; 75.1% females) and was recruited from Health Care Centres from Peking University People's Hospital. All patients and healthy controls were Han Chinese originating from northern China. The study was approved by the medical ethics committee of Peking University People's hospital and informed consents were obtained from all participants. The demographic and clinical characteristics of all subjects are summarised in Table I.

Genotyping of PADI4 *single nucleotide polymorphisms*

Genomic DNA was extracted from the peripheral blood leukocytes using a DNA extraction kit (QIAGEN microDNA, Tokyo, Japan), and then stored at -80°C. The *PADI4*_94 (rs2240340) and *PADI*4_104 (rs1748033) polymorphisms were detected by TaqMan single nucleotide polymorphism (SNP) Assays

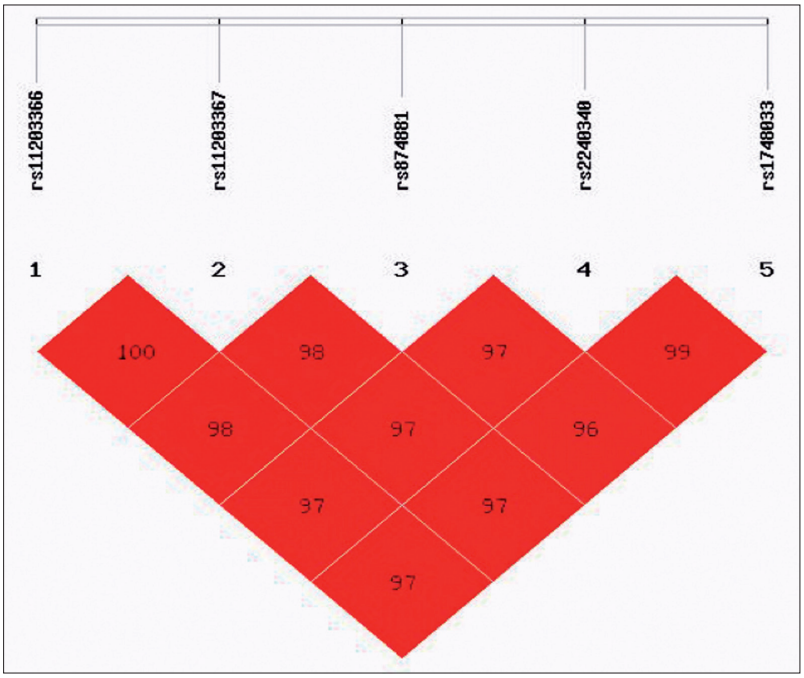

Fig. 1. Linkage Disequilibrium of 5 SNPs in PADI4. Five SNPs of exon 2, 3 and 4 from PADI4 gene were in the same block with D'>0.95.

Table I. Demographic characteristics of the study cohort.

	Controls (n=1040)	RA cases (n=1216)
Female, (%)	75.1	81.4
Age (mean ± SD years)	40.8 ± 16.3	54.4 ± 13.5
Age onset (mean ± SD years)	–	46.0 ± 14.4
Disease duration (mean ± SD years)	–	8.5 ± 8.1
RF-positive	–	78.4
CRP (mg/L)	–	30.8 ± 37.9
DAS28 Score	–	5.54 ± 1.77
Anti-CCP positive, (%)	–	82.5
SHSs (mean ± SD)	–	83.6 ± 64.7

Anti-CCP: anti-cyclic citrullinated peptide antibody; SHSs: Sharp-van der Heijde scores (SHSs) of hands.

(C_31910050_10 and C_1164586_20; Applied Biosystems). Allelic discrimination was performed using the ABI 7300 Real-Time PCR system.

Measurement of bone destruction

Radiographs were scored according to the Sharp-van der Heijde scores (SHSs) method (28). In total, 463 x-ray sets of hands were available. All x-rays were chronologically scored by one experienced radiologist who was blinded to patients' clinical and laboratory data using SHSs on hands.

Detection of serum anti-CCP antibody and anti-PAD4 antibody

The anti-CCP antibody levels were measured by enzyme-linked immunosorbent assay using the Diastat Anti-CCP kit FCCP 200, according to the recommendations of the manufacturer (Axis-Shield, Dundee, UK). Samples with results >5 RU/mL were defined as positive. The intra-and interassay coefficients of variation about the anti-CCP ELISA test were 4.0% and 6.0% respectively.

Serum anti-PAD4 levels were measured in 521 RA patients, as described by Zhao *et al*. previously (29).

Power analysis

The power analyses were performed retrospectively for the available samples (cases and controls), using a fixed minor allele frequency of 42%, a Type I error p of 0.05, and an OR of 1.40. The PS software (version 3.0.14) was used for power calculation (available at http://www.mc.vanderbilt.edu/prevmed/ps)

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was performed for each polymorphism, using Pearson's goodness-of-fit chi-square test. The Pearson chi-square tests were performed for the comparisons of allelic frequency differences between cases and controls. The odds ratios (OR) and 95% confidence intervals (CI) for alternative genetic model (dominant model) analysis were calculated using logistic regression, adjusting for age and sex. The linkage disequilibrium (LD) and haplotype were calculated using Haploview version 4.2 (http://www.broad.mit.edu/mpg/haploview/). The putative risk factors including non-genetic factors on joint damage were assessed using univariate linear regression analyses (univariate-based feature selection process). The SHSs (hands) were log-transformed to obtain a normal distribution for statistical analyses (30).

Results

Allelic frequencies of SNPs rs2240340 and rs1748033 were in Hardy-Weinberg equilibrium in both patients and controls (p>0.05). The allele frequencies of the rs2240340 (41.9%) and rs1748033 (36.3%) were similar to the data from HapMap CHB (Chinese Han Beijing, http://hapmap.ncbi.nlm.nih.gov/). The study has a statistical power of 0.978 to detect the modest effect size of OR=1.40.

Association of PADI4 *and its haplotype with RA susceptibility in a Chinese Han population*

In our cohort, both SNPs rs2240340 and rs1748033 were associated with increased susceptibility to RA at allelic level (rs2240340: OR=1.23, 95% CI 1.09–1.38, $p=6.66\times10^{-4}$; rs1748033: OR=1.24, 95% CI 1.10–1.41, $p=6.98\times10^{-4}$ respectively, Table II), which was in concordance with the results from other Asian populations (7,

Table II. Association of PAD4 SNPs with RA, adjusting for age and gender.

PADI4 SNPs		RA	Controls	*p*-value	OR (95% CI)
rs2240340		n=1216	n=1021		
Allelic	T/C	1143/1289	856/1186	6.66×10^{-4}	1.23 (1.09–1.38)
Genotypic	CT+TT/CC	876/340	660/361	2.19×10^{-4}	1.46 (1.19–1.78)
rs1748033		n=1038	n=1040		
Allelic	T/C	861/1215	756/1324	6.98×10^{-4}	1.24 (1.10–1.41)
Genotypic	CT+TT/CC	670/368	603/437	4.55×10^{-3}	1.33 (1.09–1.62)

RA: rheumatoid arthritis; *P-adj*, *p*-value adjusted by sex and age using multivariate logistic regression analysis; OR, odds ratios; CI, confidence interval.

Table III. Association of rs2240340-rs1748033 haplotypeswith RA adjusting for age and gender.

Haplotype	RA (%)	Control (%)	*p*-value	OR (95% CI)
T-T	41.2	36.5	$3.31\text{x}10^{-3}$	1.21 (1.07–1.38)
T-C	5.8	5.3	0.44	1.11 (0.85–1.46)
C-C	52.9	57.8	$1.20\ \text{x}10^{-3}$	0.81 (0.72–0.92)

RA: rheumatoid arthritis; OR (95% CI): odds ratio (95% confidence interval); variants order: rs2240340 – rs1748033.

Table IV. Association between PADI4 and anti-CCP status adjusting for sex and age.

	PADI4 SNPs (Genotype)	*p*-value	OR (95% CI)
	rs2240340 (CC /CT+ TT)		
Controls	361/660	–	Ref
Anti-CCP positive	172/503	5.13×10^{-6}	1.72 (1.36–2.18)
Anti-CCP negative	40/103	0.15	1.37 (0.90–2.09)
	rs1748033 (CC /CT+ TT)		
Controls	238/616	–	Ref
Anti-CCP positive	173/344	2.97×10^{-3}	1.44 (1.13–1.83)
Anti-CCP negative	40/76	0.23	1.31 (0.85–2.02)

RA: rheumatoid arthritis; OR (95% CI): odds ratio (95% confidence interval); Anti-CCP: Anti-cyclic citrullinated peptide antibody.

14, 17). Genotypic frequencies were also compared after adjusted for the confounding factors (sex and age). Both rs2240340 and rs1748033 displayed significant association with increased RA susceptibility at genotypic level (dominant model rs2240340: OR=1.46, 95% CI 1.19–1.78, $p=2.19\times10^{-4}$; rs1748033: OR=1.33, 95% CI 1.09–1.02, $p=4.55\times10^{-3}$; OR=1.33, 95% CI 1.09–1.62, respectively, Table II).

The two SNPs were in completely LD with D'= 0.989. Haplotypes were constructed with the two SNPs in all study subjects. Three different haplotypes were identified in this study (Table III). Two common haplotypes, TT and CC constituted almost all of the haplotypes (94.1%). Haplotype TT (41.2% of all patient haplotypes) confer the major RA risk effect (OR 1.21, 95% CI 1.07–1.38, $p=3.31\text{x}10^{-3}$). Whereas the common haplotype CC (52.9% of all patient haplotypes), confer RA protective effect (OR 0.81, 95% CI 0.72–0.92, $p=1.20\ \text{x}10^{-3}$, Table III).

PADI4 *polymorphisms conferred great risk for developing anti-CCP-positive RA*

Following stratification for anti-CCP status, we found significant association of rs2240340 and rs1748033 with anti-CCP-positive RA (OR 1.72, 95% CI 1.36–2.18, $p=5.13\times10^{-6}$ for rs2240340 and OR 1.44, 95% CI 1.13–1.83, $p=2.97\times10^{-3}$ for rs1748033, respectively, Table IV). In contrast, there was no association between the two SNPs and anti-CCP-negative RA (OR 1.37, 95% CI 0.90–2.09, $p=0.15$ for rs2240340 and OR 1.31, 95% CI 0.85–2.02, $p=0.23$ for rs1748033, respectively, Table IV). In addition, we analysed the association between *PADI4* polymorphisms and the level of anti-PAD4 antibody, however, no association was observed between two parameters (dominant model: $p=0.36$, Fig. 2).

Association between PADI4 polymorphisms and bone erosion

Bone erosion was assessed by SHSs to further clarify the influence of *PADI4* polymorphisms on disease severity. In our cohort, the univariate analysis identified several risk factors for radiographic progression, *i.e.* anti-CCP positive RA ($p=5.09\times10^{-3}$), female sex ($p=1.43\times10^{-3}$), younger age at onset ($p=5.03\times10^{-4}$) and diseases duration ($p=3.36\times10^{-23}$). A trend association between *PADI4* (rs2240340) and radiographic severity SHSs was also observed, though did not reach statistical significance ($p=0.088$, Table V).

Discussion

Meta-analysis of eastern Asian populations provides evidence of association between *PADI4* and RA susceptibility. However, the data from Chinese population were under the statistic power, with sample size less than 400 in all reports (12, 13). In present study, we conducted a case-control study involving 1216 patients with RA and 1040 controls from Chinese Han population. Our study confirms the association of *PADI4* SNPs rs2240340 and rs1748033 with RA susceptibility in Asian populations. As far as we know, this is the largest case-control study with power of 97.8% to investigate the association between *PADI4* polymorphisms and RA in Han population. PADI4 catalyses protein citrullination and the associations of *PADI4* polymorphisms with the presence or the level of anti-CCP antibody have been investigated, Positive correlations were observed both in present study and in previous studies (13, 14, 16). However, conflicting results have also been reported (20). The reason for this heterogeneous effect of *PAD4* on anti-CCP development is likely due to genetic and/or

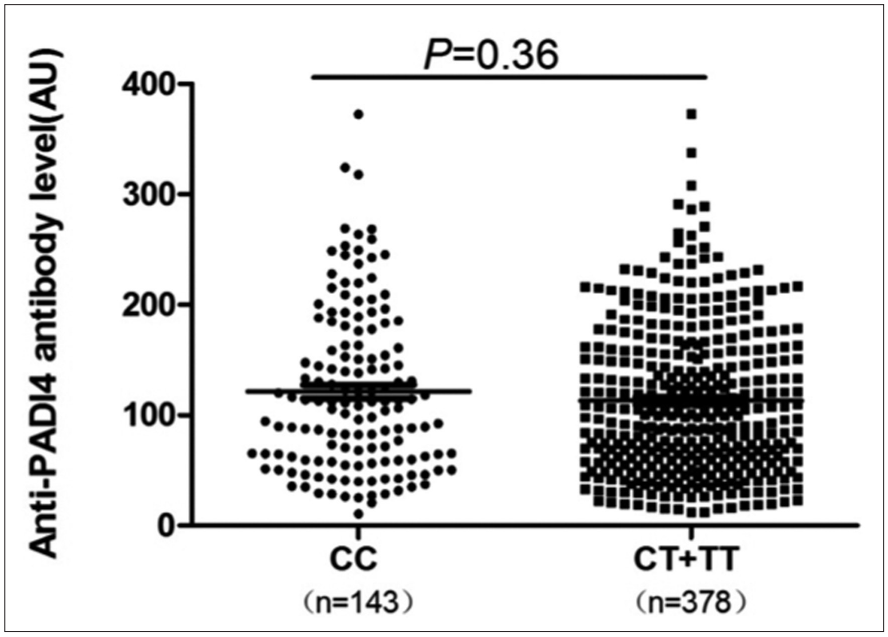

Fig. 2. Effect of PADI4 risk genotype on the production of anti-PAD4 antibody.
Serum Anti-PAD4 antibody was measured by ELISA. The anti-PAD4 level was lower in risk allele T carrier but the difference was not significant (CT+TT *vs*. CC, T test, p=0.36). Results were expressed as Mean±SEM.

Table V. Univariate linear regression analysis on putative risk factors for radiographic progression: non-genetic and genetic factors.

Putative risk/gene	n	β	*p*-value
Duration	463	0.434	3.36×10^{-23}
Anti-CCP status	441	0.131	5.09×10^{-3}
Gender (Female)	463	0.146	1.43×10^{-3}
Age onset	463	-0.159	5.03×10^{-4}
PADI4 (rs2240340)	462	0.079	0.088

Anti-CCP: anti-cyclic citrullinated peptide antibody; PADI4: peptidyl-arginine-deiminase type IV.

clinical heterogeneity between populations such as disease duration and bone erosion score. Michelle *et al*. reported that *PADI4* susceptibility haplotype had an effect on anti-PAD4 antibodies production in 111 patients with RA (31). However, in present study, we did not observe any association between *PADI4* susceptible genotype and the level of anti-PAD4 antibody in 521 patients with RA.

We chose Sharp scores instead of Steinbrocker stage to evaluate bone erosion. SHSs were continuous variable, which may provide more information than the scoring by categorical variable, *e.g*. Steinbrocker stage (10, 20). Recently, Suzuki *et al*. showed that *PADI4* risk allele was independent genetic risk for radiographic progression in 865 Japanese RA patients (19). In our 463 RA patients, we also found a suggestive association between *PADI4* risk allele and radiographic severity, though did not reach the statistic difference. It may be due to the relative smaller sample size regarding radiographic data, resulted in an insufficient power to detect the difference of bone erosion. Additional studies with larger radiographic data are needed to confirm the finding.

In present study, only SNPs from exon 2, 3 and 4 were selected since they have been extensively reported to be associated with RA both in Asians and in Caucasians (7, 21-23). Other SNPs that cover *PADI4* variability may also play a role in PAD4. Therefore, further studies are needed to establish the etiological variant involved in the susceptibility of RA.

In conclusion, our study provided strong evidence that the *PADI4* polymorphisms contribute to RA susceptibility, especially for anti-CCP positive RA, and may confer higher risk of RA radiographic severity in Chinese Han population.

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